HAYATI Journal of Biosciences 23 (2016) 125–131

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences

Original research article

Production of Xylanase by Recombinant *Bacillus subtilis* DB104 Cultivated in Agroindustrial Waste Medium

Is Helianti,[1*] Maria Ulfah,[1] Niknik Nurhayati,[1] Dadang Suhendar,[1] Anita Kusuma Finalissari,[1,2] Agustin Krisna Wardani[2]

[1] *Center for Bioindustrial Technology, Agency for Assessment and Application of Technology (BPPT), LAPTIAB-BPPT, PUSPIPTEK Serpong, Tangerang Selatan, Indonesia.*

[2] *Department of Agricultural Product Technology, Faculty of Agriculture Technology, University of Brawijaya, Malang, Indonesia.*

ARTICLE INFO

Article history:
Received 4 November 2015
Received in revised form
5 June 2016
Accepted 18 July 2016
Available online 20 August 2016

KEYWORDS:
corncobs,
cost-effective medium,
endoxylanase,
recombinant *Bacillus subtilis* DB104,
tofu liquid waste,
xylooligosaccharides

ABSTRACT

A recombinant *Bacillus subtilis* DB104 strain harbouring recombinant plasmid pSKE194 containing an Open Reading Frame (ORF) of endoxylanase and its indigenous promoter from the wild-type *B. subtilis* AQ1 strain was constructed. This recombinant *B. subtilis* DB104 strain had higher endoxylanase activity than the nonrecombinant *B. subtilis* DB104 strain in standard media, such as Luria Bertani (LB) and LB with xylan. The agroindustrial wastes corncobs and tofu liquid waste were chosen as cost-effective carbon and nitrogen sources, respectively, to test the economics of xylanase production using the recombinant *B. subtilis* DB104 at a larger scale. Submerged fermentation using a 4.5 L working volume fermentor with tofu liquid waste and 4% corncobs produced maximum xylanase activity of 1296 ± 1.2 U/mg (601.7 ± 0.6 U/mL) after 48-hour fermentation at 37°C with 150 rpm agitation; this is more than twofold higher than the activity produced in an Erlenmeyer flask. This is the first report of high xylanase activity produced from recombinant *B. subtilis* using inexpensive medium. During fermentation, the xylanase degrades corncobs into xylooligosaccharides, showing its potential as an enzyme feed additive or in xylooligosaccharide production.

1. Introduction

The majority of agroindustrial waste is lignocelullosic biomass containing xylan, which is the second most abundant polysaccharide in the world (Beg *et al.* 2001). It is the source of several valuable xylan derivatives, such as xylose and xylooligosaccharides (XOS) (Vazquez *et al.* 2000; Akpinar *et al.* 2007), as well as a potential feedstock for lignocellulosic biomass-based bioethanol. Xylanases are important enzymes that are often used to transform xylan into its derivatives. They are also used to generate renewable fuels and chemicals from agricultural lignocellulosic materials. They are also used in the paper and pulp industries, where xylanases can reduce the environmental burden or as feed enzymes (Kulkarni *et al.* 1999; Subramaniyan and Prema 2002; Wu *et al.* 2005; Motta *et al.* 2013). XOS derived from xylanase-degraded xylan can be used as functional foods/feed additives (i.e. prebiotics) or further transformed to xylitol and used as an alternative sweetener (Vazquez *et al.* 2000; Motta *et al.* 2013). Cheaper enzymes are currently required for any industrial application. Increasing enzyme expression or secretion via genetic engineering would improve the economics of these processes. In addition, engineering of fermentation processes using cost-effective media components, such as agroindustrial waste and/or optimising fermentation conditions such as agitation, aeration, pH, and temperature condition, are other means to improving economic value (Motta *et al.* 2013; Prasad *et al.* 2014).

The bioconversion of agroindustrial wastes to high-value products would not only recover value from waste but would also mitigate some environmental issues such as disposal management and pollution. For example, 19 million tonnes of corn, which is one of Indonesia's agroindustrial plants, were produced there in 2014 (http://www.bps.go.id/Brs/view/id/1122). Clearly, Indonesia has abundant agricultural biomass as a by-product, which, if left unprocessed, poses a serious environmental problem. Corncobs contain significant amounts of xylan (Barl *et al.*1991), making it an ideal substrate for xylanase production via microbial fermentation.

* Corresponding author.
E-mail addresses: ishelianti@gmail.com, is.helianti@bppt.go.id (I. Helianti).
Peer review under responsibility of Institut Pertanian Bogor.

http://dx.doi.org/10.1016/j.hjb.2016.07.002

Traditional tofu industries generate liquid waste that can also become a serious environmental pollution problem. Although tofu liquid waste (TLW) is considered a high protein source for potential use in fermentation medium, only a small percentage of the waste has been utilised thus far and the rest remains industrial waste (Kim and Lee 2010). Several reports have described the utilisation of agroindustrial wastes as potential components for fermentation medium (De Azeredo *et al.* 2006; Prakasham *et al.* 2005); however, none of these publications used recombinant microbes.

We have previously reported the cloning and expression of endoxylanase genes from *Bacillus subtilis* AQ1 in *Escherichia coli* (Helianti *et al.* 2010). The recombinant xylanase had optimum growth at pH 7 and 55°C, but there was still significant activity at 37°C and lower pH levels. These properties make it suitable for application as an enzyme feed additive to improve broiler performance (Wu *et al.* 2005). In this study, we have subcloned and expressed this xylanase gene in *B. subtilis* DB104 using a shuttle *E. coli-Bacillus* vector. This study demonstrates the production of xylanase by this recombinant *B. subtilis* using low cost carbon (corncobs) and nitrogen (TLW) sources to reduce the cost of enzyme production and provide an alternative to costly disposal of crop biomass and agroindustrial wastes.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and standard media

The bacterial strains used were *E. coli* DH5α (purchased from ITB, Bandung) as a host for the recombinant plasmid propagation and *B. subtilis* DB104 for subcloning and expression. We used a pSKE194 plasmid as an *E. coli-Bacillus* shuttle vector (*B. subtilis* DB104 strain, and the plasmid were provided by Prof Meinhardt, Muenster University). All antibiotics used in the experiments were from Sigma, St. Louis, Missouri, USA, and the media components, such as yeast, tryptone, and agar, were from Difco, New Jersey, USA. To regenerate *Bacillus* protoplasts containing the desired recombinant plasmid, 1.5% (w/v) agar containing regeneration medium DM3 was used (Chang and Cohen 1979). To select positive *Bacillus* transformants, LB medium containing erythromycin 5 μg mL^{-1} was used. For confirmation of gene expression, LB agar medium containing ampicillin (100 μg mL^{-1}) or erythromycin (5 μg mL^{-1}) and 0.7% (w/v) oat spelt xylan (Roth, Germany) was used.

2.2. Subcloning of the xylanase gene in the *Bacillus-E. coli* shuttle vector

All genetic engineering experiments were carried out based on standard protocols (Sambrook and Russel 2001). The inserted endoxylanase gene (*xyn11*AQ1) with confirmed DNA sequence was isolated from the previously reported recombinant pGEM-T easy plasmid (Helianti *et al.* 2010) by restriction digest with *Kpn*I and *Bgl* II. The gene was then ligated into the pSKE194 vector at the *Kpn* I and *BamH* I sites because the vector has no *Bgl* II as a unique site and *BamH* I and *Bgl* II have compatible ends.

The recombinant plasmid was transformed into *E. coli* DH5α. Xylanase activity was observed as xylan–Congo red clearance on oat spelt xylan-LB agar. The positive clones harbouring recombinant pSKE 194 plasmids were selected as clear zone-forming colonies on oat spelt xylan-LB agar medium. The recombinant pSKE194-*xyn11*AQ1 plasmid was then extracted from *E. coli* and used to transform *B. subtilis* DB104 using protoplast transformation. *Bacillus* protoplasting and transformation were performed based on the method by Chang and Cohen (1979). The positive transformants were chosen by selection on erythromycin resistance medium.

2.3. Confirmation of the increased endoxylanase activity in recombinant *B. subtilis* DB104

The erythromycin-resistant transformants were selected from erythromycin-containing LB medium and cultivated in liquid LB medium containing the same antibiotic. The plasmid was extracted from liquid culture (Voskuil and Chambliss 1993) and analysed by restriction digest. Xylanase activity of the positive transformants was qualitatively checked on LB xylan agar medium by measuring the clear zone ratio [clear zone ratio = diameter of clear zone (cm)/diameter of the colony (cm)]. Nonrecombinant *B. subtilis* DB104 was used as a control.

2.4. Enzyme production by recombinant *B. subtilis* DB104 cultivated in standard medium

One single colony of recombinant *B. subtilis* DB104 was inoculated into both 10-mL LB medium and LB 1% xylan medium. The cultures were grown in the presence of erythromycin until the OD_{600} reached 0.95, and then 2.5 mL of this preculture was used to inoculate a fresh 100 mL of LB or LB-xylan medium. The new culture was cultivated at 37°C in a shaker incubator (Kuehner, Germany) with 150 rpm agitation. Bacterial number was measured by the spectrophotometric method and plating (colony forming unit method). Samples were taken from the culture every 4 hours during the 32-hour fermentation to test bacterial number, endoxylanase activity, and protein concentration. The same procedure was applied to the nonrecombinant *B. subtilis* DB104.

2.5. Preparation of corncobs and tofu waste liquid

Corncob was purchased from the Agrotechnology Business Area (Kawasan Agroteknobisnis Sumedang), Sumedang, West Java. They were crushed, ground, and sieved into particles with different mesh sizes (20, 40, 70, and 100). Various sizes of corncobs were used in Erlenmeyer flask scale and thin layer chromatography (TLC) analyses; however, only corncobs with 20 mesh size were used in production in the fermentor. TLW was obtained from a tofu manufacturer at Serpong, Tangerang Selatan, Banten. The range of total protein content in TLW, measured by the Kjedahl method (Kirk 1950), was between 0.003 and 0.02% in the water-containing form. The glucose concentration (detected by a glucose sensor) was between 10 mg/dL and 140 mg/dL, and the pH was 4–6.

2.6. Selection of cost-effective medium

The substitution of xylan with corncobs was conducted after confirming the increased activity in LB-xylan medium. Different corncob concentrations (1%–5%) were tested in LB medium, and the growth, activity, and protein concentration were observed. Then, the medium with the corncob composition giving maximum xylanase production was chosen for further tests. TLW and organic [mixture of peptone (0.25%) and yeast extract (0.15%)] and inorganic nitrogen sources [$(NH_4)_3PO_4$ 0.93%, $(NH_4)_2SO_4$ 0.93%, urea 0.42%, and NH_4NO_3 0.56%] were tested, and the nitrogen source that gave the highest activity was chosen for further tests.

2.7. Production of endoxylanase using recombinant *B. subtilis* DB104 in alternative cost-effective medium at Erlenmeyer flask scale

Medium containing TLW, 4% corncobs sieved with size 20 mesh, and 1% NaCl was used at Erlenmeyer flask scale and larger scale fermentation. TLW was used without any dilution. The initial pH of the medium was adjusted to 7, and the medium was autoclaved at 121°C for 15 minutes. One single colony of recombinant *B. subtilis* DB104 was inoculated into 10-mL LB in the presence of erythromycin until the OD_{600} reached 0.95, and then 2.5 mL of this preculture was used to inoculate a fresh 100 mL of cost-effective

medium. The fermentation was performed at 37°C for 28 hours at 150 rpm agitation.

2.8. Endoxylanase production with submerged fermentation (4.5 L working volume) in a 6-L fermentor

Larger scale fermentation tests were performed in a 6-L fermentor (LKB, Germany) with a working volume of 4.5 L. Several aeration [0.4; 0.45; and 0.6 vvm (gas volume flow per unit of liquid volume per minute = volume per volume per minute)] and agitation (130 and 150 rpm) rates were tested to explore the maximum endoxylanase activity. The protein, glucose content, and pH of every batch of TLW was measured before use. A 450-mL culture in LB medium with OD_{600} ~0.8 was inoculated into a 4.05-L mixture medium of TLW, 4% corncobs, and 1% NaCl. The fermentation was run for 54 hours, and the samples were taken every 6 hours. The bacterial cell concentration was measured by plating (colony forming unit method).

2.9. Enzyme and protein assay

Crude xylanase extract was obtained by recovering supernatant after centrifuging the culture (3000 ×*g*, 10 minutes, 4°C). This crude enzyme extract was used for enzyme assays. Xylanase activity was measured (each sample in triplicate) by the Miller method using dinitrosalicylic acid to detect reducing sugar with D-xylose as a standard (Bailey *et al.* 1992; Miller 1959); this procedure has been previously described (Helianti *et al.* 2010). Protein concentration was measured by the dye-binding assay method with bovine serum albumin as the standard protein (Bradford 1976).

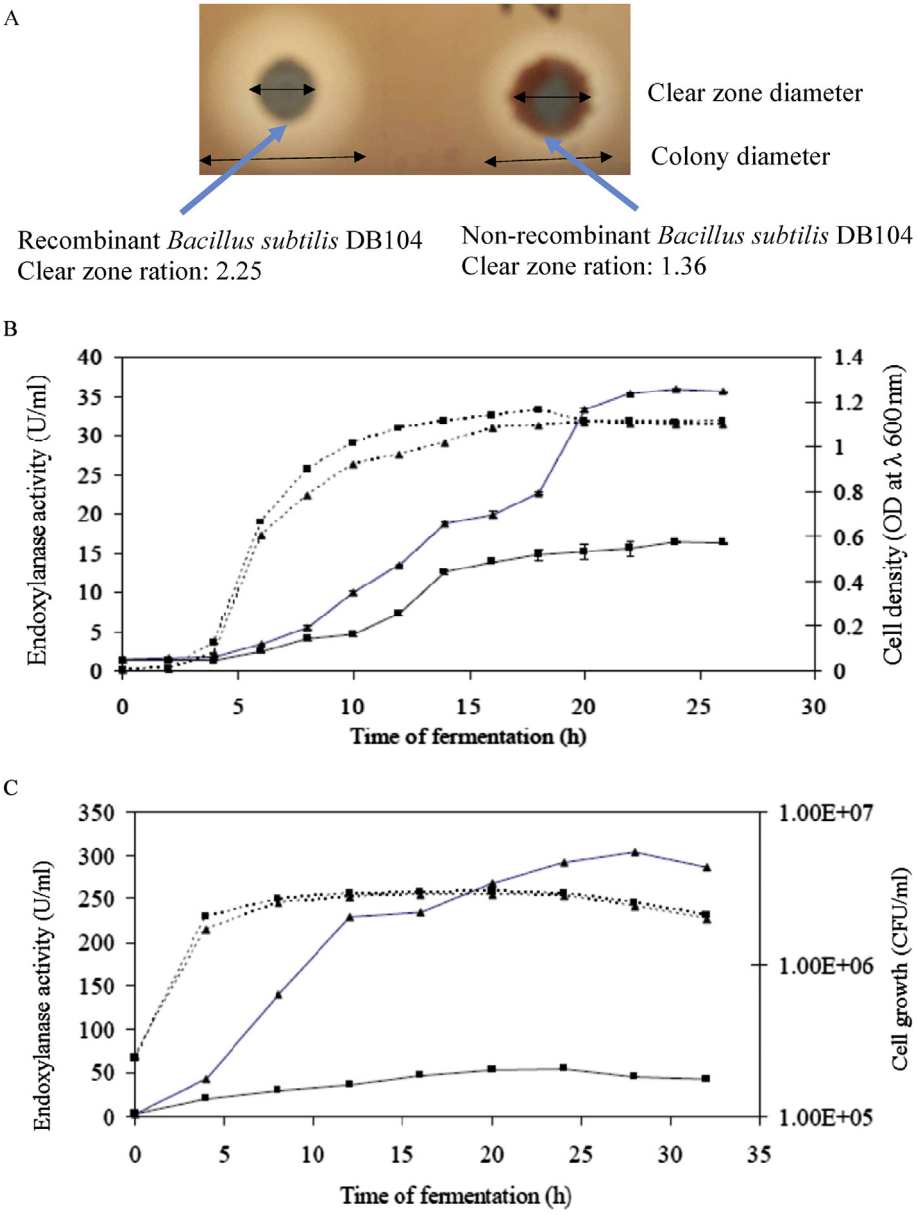

Figure 1. Production of endoxylanase in different defined media using recombinant and nonrecombinant *Bacillus subtilis* DB104. (A) Comparison of the clear zone ratio between recombinant and nonrecombinant *B. subtilis* DB104 in LB-xylan agar medium. The clear zone ration is defined as the clear zone diameter (cm)/colony diameter (cm) (Arrows pointed out each colony of B. subtilis DB104). (B) Comparison of xylanase production between nonrecombinant and recombinant *B. subtilis* in LB medium. (C) Comparison of xylanase production between nonrecombinant and recombinant *B. subtilis* in LB xylan medium. Triangle with bold line—▲—: the activity from recombinant *B. subtilis*; square with bold line —■—: the activity from the nonrecombinant one; triangle with dot line– ▲ – : growth of recombinant *B. subtilis*; square with dot line-■- -: growth of the nonrecombinant one.

2.10. Analyses of fermentation products during fermentation using thin layer chromatography

Approximately, 0.75 μL of fermentation broth sampled at 0, 8, 24, and 32 hours was dropped on TLC plates coated with a 0.2-mm layer of silica gel G. XOS standards [xylose, xylobiose, xylotriose, and xylotetraose (Sigma, USA)] were also treated in the same manner. The plate was developed with a solvent system of butanol, acetic acid, and water at a ratio of 2:1:1 (v/v). The staining mixture contained aniline, diphenylamine, acetone, and phosphate acid (0.4 mL, 0.4 g, 20 mL, and 3 mL, respectively). The TLC plate was dried for 4 hours at 60°C (using an oven) until spots appeared.

3. Results

3.1. Xylanase activity of recombinant *B. subtilis* and nonrecombinant *B. subtilis* in standard media at Erlenmeyer flask scale

Subcloning of the endoxylanase gene and its promoter from *B. subtilis* AQ1 into the *Bacillus-E. coli* pSKE194 shuttle vector was conducted, and the recombinant plasmid was introduced into *B. subtilis* DB104. The newly constructed recombinant *B. subtilis* DB104 harbouring the *B. subtilis* AQ1 xylanase gene (pSKE194-*xyn11*AQ1) showed a higher clear zone ratio (2.25) on LB-xylan agar compared to the nonrecombinant strain (1.36) (Figure 1A).

The expression of the two homologous endoxylanase genes, one in the *B. subtilis* DB104 chromosome and the other one in the plasmid pSKE194 (originating from *B. subtilis* AQ1), was constitutive. Even when there was no substrate (xylan) in the medium, xylanase activity was still observed at a lower level (Figure 1B). The maximum xylanase activity of recombinant *Bacillus* in LB medium (100-mL fermentation in Erlenmeyer flasks) was achieved after 24 hours when the cell density or absorbance reached 1.2 at λ 600 nm. The maximum xylanase activity produced by recombinant *B. subtilis* in LB medium was 35.9 U/mL, which is more than two times higher than the nonrecombinant strain (16.5 U/mL) (Figure 1B).

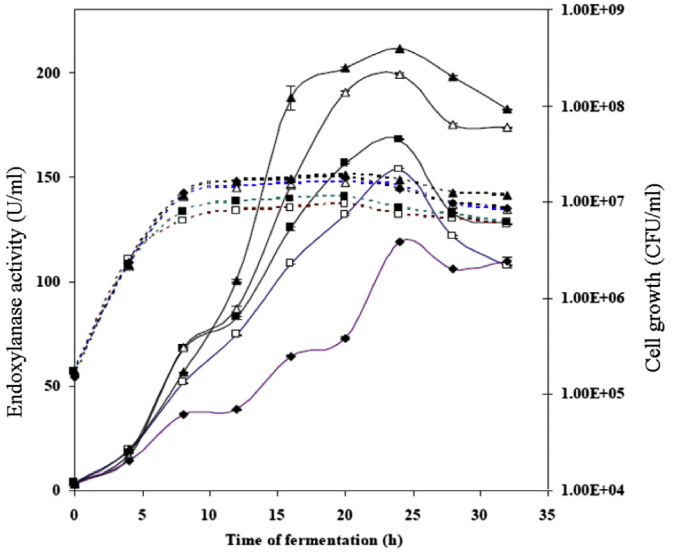

Figure 2. Effect of different corncob concentrations on the production of xylanase using recombinant *Bacillus subtilis* DB104 in LB medium. (—□—): showing the activity coming from recombinant *B. subtilis* DB104 using 1% corncobs; (—■—): 2% corncobs; (—△—): 3% corncobs; (—▲—): 4% corncobs; (—◆—): 5% corncobs. (- -□- - -): showing growth of recombinant *B. subtilis* DB104 using 1% corncobs; (- - -■- - -): growth using 2% corncobs; (- - -△- - -): growth using 3% corncobs; (- - -▲- - -): growth using 4% corncobs; (- -◆- -): growth using 5% corncobs.

However, when cultivated in LB-oat spelt xylan medium, the recombinant *B. subtilis* DB104 strain produced more than seven times the activity of the nonrecombinant strain (302.6 U/mL compared to 42.6 U/mL). This occurred at 28 hours with similar bacterial growth curves (Figure 1C) and shows that the presence of xylan induces the productivity of endoxylanase in both recombinant and nonrecombinant *B. subtilis*.

3.2. Selection of cost-effective medium

After several trials in Erlenmeyer flask experiments using several media, it is clear that the recombinant *B. subtilis* has consistently higher endoxyalanase activity than the nonrecombinant strain (data not shown). To remove the expensive components, such as xylan, yeast, and tryptone, which are unavoidable when standard medium is used, several cost-effective media were tested. Corncobs containing 32% xylan (data not shown) were used to replace the expensive carbon source, xylan. To find the suitable corncob concentration yielding the best endoxylanase activity, small-scale fermentations were conducted using 1%–5% corncob-containing LB in Erlenmeyer flasks. The xylanase activity increased with the corncob concentration up to 4%. However, at 5% corncob, the xylanase activity decreased. Thus, 4% corncobs provided the best endoxylanase activity (211 U/mL) after 24 hours (Figure 2). The level of endoxylanase activity of recombinant *B. subtilis* grown in a medium containing corncobs was higher than that grown in LB; this indicates that xylan-rich natural substrates, such as autoclaved corncobs, can induce xylanase productivity.

The medium containing corncobs was then further modified by replacing the expensive nitrogen sources (i.e. peptone, tryptone, and yeast extract) with TLW. Before choosing TLW, we tried several inorganic and organic nitrogen sources. However, when cultivated in medium containing these nitrogen sources, the recombinant *B. subtilis* strain produced lower xylanase activity than in LB medium (data not shown). When the yeast and tryptone in the medium were replaced with TLW, the xylanase activity was 226 U/mL (Figure 3). Thus, the combination of TLW and 4% corncobs was selected as a suitable medium for larger scale fermentation.

3.3. Cultivation in a 6-L scale fermentor with 4.5-L working volume

We performed larger scale tests to investigate the feasibility of the medium for further applications. Our experiments using corncobs with different particle sizes showed that the size did not significantly impact activity (data not shown). Therefore, we used

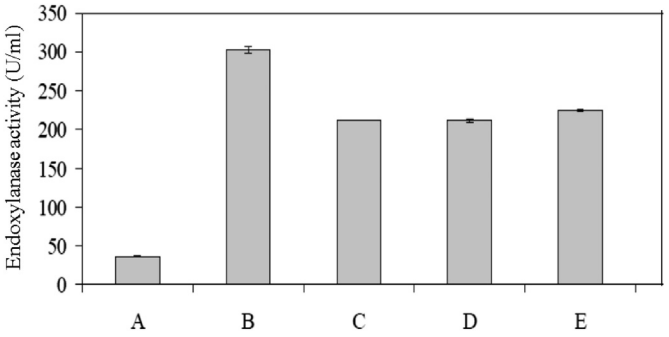

Figure 3. Comparison of endoxylanase activity in an Erlenmeyer flask using different media: (A) endoxylanase productivity using LB medium; (B) endoxylanase productivity using LB-xylan medium; (C) endoxylanase productivity using LB medium containing 4% corncobs; (D) endoxylanase productivity using 1% xylan and TLW; and (E) endoxylanase productivity using 4% corncobs and TLW. All values are the means of triplicates, and error bars indicate the standard deviation of independent experiments. TLW = tofu liquid waste.

the most affordable size (20 mesh) for enzyme production. The parameters used in the 4.5-L fermentation were determined based on the Erlenmeyer flask experiments and were 4% corncob concentration, initial medium pH 7, and at least 0.005% nitrogen content of TLW. The initial pH of the medium was critical for the fermentation, as we observed that there was no bacterial growth if the initial pH of the medium was below 5. During fermentation, pH was monitored but not tightly controlled. We found that, to give good conditions for fermentation, the minimal protein content of TLW needed to be at least 0.005% (w/v). Good cellular growth and enzyme activity could not be obtained when the protein content of TLW was below 0.005% or more than 0.02%.

Maximum xylanase production was obtained when the fermentation was carried out at 37°C with an aeration rate of 0.6 vvm, 150 rpm agitation, density of the starter OD_{600} of 0.8, and an initial glucose concentration of 143 mg/dL. Under these conditions, the maximum activity of 1296 ± 1.2 U/mg or 601.7 ± 0.6 U/mL could be reached after 48 hours (Table 1).

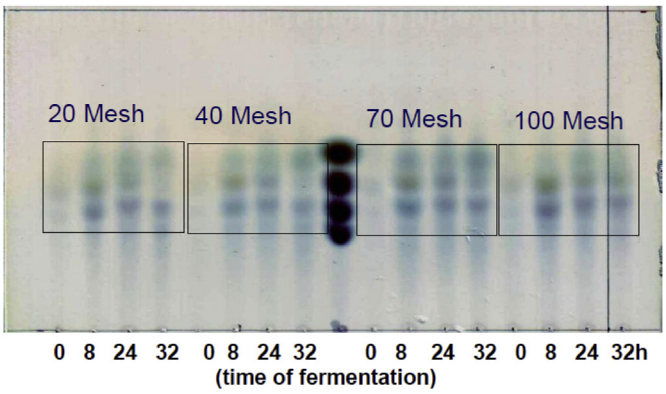

Figure 4. Analyses of fermentation products from Erlenmeyer flasks using 4% concentrations of different corncob sizes using thin layer chromatography (TLC) after 0, 8, 24, and 32 hours of fermentation. The middle black blot is the xylooligosaccharide standard, from top to bottom: xylose, xylobiose, xylotriose, and xylotetraose.

3.4. Analyses of the metabolic products during fermentation using corncobs and TLW

At the beginning of fermentation (zero hour), the presence of XOS could not be detected. As the fermentation process progressed, the presence of XOS (xylose, xylobiose, xylotriose, and xylotetraose) became apparent (Figure 4). The size range (20–100 mesh) of the corncobs used in the fermentation did not impact the XOS produced. At the end of fermentation, XOS was still detected (i.e. not degraded into xylose). The TLC results confirm that the enzyme was endoxylanase, which has potential applications for XOS production from lignocellulosic biomass.

4. Discussion

We successfully subcloned and expressed the endoxylanase gene and its promoter from *B. subtilis* AQ1 into the *Bacillus-E. coli* pSKE194 shuttle vector in *B. subtilis* DB104. The higher endoxylanase activity of recombinant *B. subtilis* DB104 showed that the DNA recombinant technology indeed enhanced the xylanase production. Previously, Jeong *et al.* (1998) described the expression of an endoxylanase gene from *Bacillus* sp., cloned in pJHKJ4 and expressed in *B. subtilis* DB104, with a maximum endoxylanase activity of 105 U/mL in the supernatant of the recombinant bacterial culture. Our results show that the presence of xylan induced the productivity of endoxylanase both in recombinant and nonrecombinant *B. subtilis*. The recombinant plasmid pSKE 194-*xyn11*AQ1 is the combination of pE194, pBluescript SK+ plasmid, and the fragment *xyn11*AQ1. The pE194 plasmid can be replicated more than 10 copies in each *Bacillus* cell (Weisblum *et al.* 1979). Therefore, more xylanase protein would be expressed in recombinant culture even though the cell density was equal to that of the nonrecombinant culture. Furthermore, the presence of substrate in xylan-containing medium is known to induce xylanase productivity (Motta *et al.* 2013). *B. subtilis* DB104 has an intrinsic chromosomal xylanase gene that is induced by xylan. The recombinant bacteria have both the intrinsic xylanase gene as well as the one inserted in the plasmid. Therefore, it is most likely that the effect of xylan was greater on the recombinant *B. subtilis*.

Subcloning and expression of extracellular protein genes in *B. subtilis* is a very promising tool for enzyme production because *B. subtilis* is known to be a safe and suitable host for extracellular enzyme production. *B. subtilis* is also a Generally Recognized As Safe microbe. Many reports have already described the advantages of using the genus *Bacillus* as an industrial workhorse for extracellular enzyme production and have reported improvement of *Bacillus* strains for increased expression efficiency (Nahrstedt *et al.* 2005; Schallmey *et al.* 2004; Waldeck *et al.* 2007).

In our study, the optimal corncob concentration was 4% (Figure 2). Lower concentrations (1%, 2%, and 3%) resulted in lower xylanase activity, which was probably due to the lower availability of nutrients. The higher concentration of corncob (5%), however, also gave lower xylanase activity, which may have been due to insufficient dissolved oxygen in the concentrated culture medium. The 4% corncob concentration may have provided ideal conditions in terms of nutrients and oxygen supply. The same phenomenon was also found in xylanase production by *Aspergillus niger*, where the optimal corncob concentration for growth and productivity was 3% (Ahmad *et al.* 2012). Activity decreased above this, probably due to the decline of nutrients in the medium or the inhibitory effects of the enzyme degradation product (i.e. XOS) (Ahmad *et al.* 2012).

The TLC analyses of fermentation broth from Erlenmeyer flask experiments showed that XOS was accumulated during fermentation. The XOS was not degraded into xylose because most of the xylanolitic enzymes secreted were in the form of endoxylanase, which only degrades xylan into XOS. This is similar to the results reported by Jeong *et al.* (1998). The size of the corncob particles for optimal production was between 20 and 100 mesh, as this range did not have any significant differences in xylanase activity or XOS production. It is possible that submerged fermentation is more flexible with regard to the size of substrate particles. In solid-state fermentation, the particle size of the substrate significantly alters the enzyme production because the substrate also becomes the

Table 1. Summary of fermentation conditions and the maximum activity at 4.5-L working volume of fermentation

Condition label	Cell density of starter at absorbance λ 600 nm	Flow rate (vvm)	Corncobs concentration (%)	TLW		Agitation (rpm)	Maximum endoxylanase activity (U/mL)
				Protein content (w/v)	Glucose content (mg/dL)		
a.	0.842	0.4	4	0.0053%	22	130	209.3 ± 0.5 (354.3 U/mg)
b.	0.840	0.45	4	0.0059%	133	150	448.3 ± 0.6 (1026.4 U/mg)
c.	0.883	0.45	4	0.0059%	143	150	481.8 ± 3.5 (1088.2 U/mg)
d.	0.843	0.6	4	0.0059%	143	150	601.7 ± 0.6 (1296 U/mg)

Table 2. Comparison of several studies related to xylanase production using different wastes and different enzyme microbial producers

Study	The medium used	The microbial producer	Activity of xylanase
This study	Medium containing 4% corncobs, tofu liquid waste, and NaCl 1%	Recombinant *Bacillus subtilis* DB104 containing pSKE-*xyn11*AQ1	601 U/mL
Lai *et al.* 2015	Medium containing pulp and paper sludges and corn stover	*Bacillus pumilus*	37.8 U/mL
Seo *et al.* 2014	Minimal nutrient medium containing copra meals	*Bacillus licheniformis*	0.34 U/mL
Kapoor *et al.* 2008	DL-β-phenylalanine, wheat bran, yeast extract, and pepton	*Bacillus pumilus* strain MK001	2886 U/mL
Dobrev *et al.* 2007	Nutrient medium containing (g/L): $(NH_4)_2HPO_4$ 2.6, urea 0.9, corncobs 24.0, wheat bran 14.6, and malt sprout 6.0.	*Aspergillus niger* B03	996.30 U/mL
Ninawe and Kuhad 2005	Medium with wheat bran as carbon source	*Streptomyces cyaneus* SN32	710 U/mL

place for proliferation of microbes (Renge *et al.* 2012). Thus, it may be possible to simultaneously produce high levels of endoxylanase and high-value XOS in the same process with these "zero value" wastes and directly reduce the production costs.

The same agricultural waste has also been used for xylanase production via fermentation using some xylan-degrading wild-type fungi (Dobrev *et al.* 2007; Ninawe and Kuhad 2005). There have been no reports regarding the use of these wastes in fermentation using recombinant *Bacillus.* High xylanase production by *Bacillus pumilus* in an alternative medium has been reported previously (Kapoor *et al.* 2008); however, expensive peptone and yeast were used as the nitrogen sources and different agricultural wastes (such as wheat bran or wheat straw) were used as carbon sources. Other groups have reported the production of xylanase by wild-type *Bacillus licheniformis* using minimal nutrient medium containing copra meals (Seo *et al.* 2014) and *B. pumilus* using a medium containing pulp and paper sludges and corn stover (Lai *et al.* 2015). However, xylanase activity was low in these studies (Table 2). We believe that our study is the first to report the production of high-level xylanase from recombinant bacteria cultivated in agricultural waste–containing medium.

The cost-effective production of this high-titre xylanase at a larger scale is promising for use as a feed enzyme. Our xylanase had activity at 37°C but was relatively heat stable and active at a low pH (Helianti *et al.* 2010), so it is applicable for feed enzyme. Hydrolysing both soluble and insoluble antinutrient nonstarch polysaccharides by xylanase improves growth performance (Kiarie *et al.* 2014). XOS is also a possible prebiotic feed additive (Hajati and Rezaei 2010). Therefore, the use of the products from this fermentation (xylanase and XOS) in poultry feed additives is promising.

In conclusion, the *B. subtilis* AQ1 endoxylanase gene with its endogenous promoter was cloned and expressed in *B. subtilis* DB104 via a shuttle plasmid. After exploring the conditions for endoxylanase production by this recombinant organism in a fermentor with 4.5-L working volume with the medium containing TLW and 4% corncob, the production was optimised and reached higher values compared to production in Erlenmeyer flasks. This sophisticated genetic engineering technology, combined with the use of low-cost medium such as TLW and corncobs, can lead to a considerable reduction in the cost of xylanase production for various industry applications, such as enzyme feed additives. At the same time, the use of these wastes will help alleviate problems with agroindustrial waste disposal in Indonesia and prevent further environmental pollution.

Acknowledgements

The authors thank Professor Dr. F. Meinhardt, Muenster University, for valuable discussion and precious gifts of materials through the IG-Biotech Collaboration; Astutiati Nurhasanah for critically reading the manuscript; and Lina Mulyawati for technical assistance.

Funding Statement

Part of this work was supported by the Incentive Research Grant 2009–2010 from the Indonesia Ministry of Research and Technology, Republic of Indonesia (No. KP-2010-2508), granted to Is Helianti as a Principal Researcher.

References

Ahmad Z, Butt MS, Anjum FM, Awan MS, Rathore HA, Nadeem MT, Ahmad A, Khaliq A. 2012. Effect of corn cobs concentration on xylanase biosynthesis by Aspergillus niger. *Afr J Biotechnol* 11:1674–82. http://dx.doi.org/10.5897/AJB11.1769.

Akpinar O, Kavas A, Bakir U, Yilmaz L. 2007. Enzymatic production of xylooligosaccharides from cotton stalks. *J Agric Food Chem* 55:5544–51.

Bailey MJ, Biely P, Poutanen K. 1992. Interlaboratory testing for assay of xylanase activity. *J Biochtenol* 23:257–70.

Barl B, Billiaderis CG, Murray D, MacGregror AW. 1991. Combined chemical and enzymatic treatments of corn husks lignocellulosic. *J Sci Food Agri* 56:195–214. http://dx.doi.org/10.1002/jsfa.2740560209.

Beg QK, Kapoor M, Mahajan L, Hoondal GS. 2001. Microbial xylanases and their industrial applications: a review. *Appl Microbiol Biotechnol* 56:326–38.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–54.

Chang S, Cohen SN. 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Mol Gen Genet* 168:111–5.

De Azeredo LAI, De Lima MB, Coelho RRR, Freire DMG. 2006. A low-cost fermentation medium for thermophilic protease production by *Streptomyces* sp. 594 using feather meal and corn steep liquor. *Curr Microbiol* 53:335–9.

Dobrev GT, Pishtiyski IG, Stanchev VS, Mircheva R. 2007. Optimization of nutrient medium containing agricultural wastes for xylanase production by *Aspergillus niger* B03 using optimal composite experimental design. *Bioresour Technol* 98:2671–8.

Hajati H, Rezaei M. 2010. The application of prebiotics in poultry production. *Int J Poult Sci* 9:298–304.

Helianti I, Nurhayati N, Ulfah M, Wahyuntari B, Setyahadi S. 2010. High level of constitutive expression of endoxylanase gene from newly isolated *Bacillus subtilis* strain AQ1 cloned in *Escherichia coli*. *J Biomed Biotechnol*. http://dx.doi.org/10.1155/2010/980567.

Jeong KJ, Park IY, Kim MS, Kim SC. 1998. High-level expression of an endoxylanase gene from *Bacillus sp.* in *Bacillus subtilis* DB104 for the production of xylobiose from xylan. *Appl Microbiol Biotechnol* 50:113–8.

Kapoor M, Nair LM, Kuhad RC. 2008. Cost-effective xylanase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of *Prosopis juliflora*. *Biochem Eng J* 38:88–97.

Kiarie E, Romero LF, Ravindran V. 2014. Growth performance, nutrient utilization, and digesta characteristics in broiler chickens fed corn or wheat diets without or with supplemental xylanase. *Poult Sci* 93:1186–96. http://dx.doi.org/10.3382/ps.2013-03715.

Kim MS, Lee DY. 2010. Fermentative hydrogen production from tofu-processing waste and anaerobic digester sludge using microbial consortium. *Bioresour Technol* 101:S48–52.

Kirk PL. 1950. Kjeldahl method for total nitrogen. *Anal Chem* 22:354–8. http://dx.doi.org/10.1021/ac60038a038.

Kulkarni N, Shendye A, Rao M. 1999. Molecular and biotechnological aspects of xylanases. *FEMS Microbiol Rev* 23:411–56.

Lai TT, Pham TTH, Adjalle K, Brouilette F, Barnabe S. 2015. Strategies for using pulp and paper sludges as culture media for xylanase production with *Bacillus pumilus*. *Waste Biomass Valor* 6:1103–13. http://dx.doi.org/10.1007/s12649-015-9404-01.

Miller GL. 1959. Use of dinitrosalycylic acid as reagent for the determination of reducing sugars. *Anal Chem* 31:208–18.

Motta FL, Andrade CCP, Santana MHA. 2013. A review of xylanase production by the fermentation of xylan: classification, characterization and applications. *Intech*: 251–75. http://dx.doi.org/10.5772/53544.

Nahrstedt H, Waldeck J, Gröne M, Eichstädt R, Feesche J, Meinhardt F. 2005. Strain development in *Bacillus licheniformis*: construction of biologically contained mutants deficient in sporulation and DNA repair. *J Biotechnol* 119:245–54.

Ninawe S, Kuhad RC. 2005. Use of xylan-rich cost effective agro-residues in the production of xylanase by *Streptomyces cyaneus* SN32. *J Appl Microbiol* 99: 1141–8.

Prakasham RS, Rao CS, Rao RS, Sarma PN. 2005. Alkaline protease production by an isolated *Bacillus circulans* under solid-state fermentation using agroindustrial waste: process parameters optimization. *Biotechnol Prog* 21:1380–8.

Prasad R, Abraham TK, Nair AJ. 2014. Scale up of production in a bioreactor of a halotolerant protease from moderately halophilic *Bacillus* sp. isolated from soil. *Braz Arch Biol Technol.* 57:448–55 http://dx.doi.org/10.1590/S1516-89132014005000014.

Renge VC, Khedkar SV, Nandurkar NR. 2012. Enzyme synthesis by fermentation method: a review. *Sci Revs Chem Commun* 2:585–90.

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd edition. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press

Schallmey M, Singh A, Ward OP. 2004. Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol* 50:1–17.

Seo J, Park TS, Kim JN, Ha JK, Seo S. 2014. Production of endoglucanase, beta-glucosidase and xylanase by *Bacillus licheniformis* grown on minimal nutrient medium containing agriculture residues. *Asian-Australas J Anim Sci* 27:946–50. http://dx.doi.org/10.5713/ajas.2014.14082.

Subramaniyan S, Prema P. 2002. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Crit Rev Biotechnol* 22:33–64.

Vazquez MJ, Alonso JL, Domnguez H, Parajo JC. 2000. Xylooligosaccharides: manufacture and applications. *Trends Food Sci Technol* 11:387–93.

Voskuil MI, Chambliss GH. 1993. Rapid isolation and sequencing of purified plasmid DNA from Bacillus subtilis. *Appl Environ Microbiol* 59:1138–42.

Waldeck J, Meyer-Rammes H, Wieland S, Feesche J, Maurer KH, Meinhardt F. 2007. Targeted deletion of genes encoding extracellular enzymes in *Bacillus licheniformis* and the impact on the secretion capability. *J Biotechnol* 130:124–32.

Weisblum B, Graham MY, Gryczan T, Dubnau D. 1979. Plasmid copy number control: isolation and characterization of high-copy-number mutants of plasmid pE194. *J Bacteriol* 137:635–43.

Wu Y, Lai C, Qiao S, Gong L, Lu W, Li D. 2005. Properties of Aspergillar xylanase and the effects of xylanase supplementation in wheat-based diets on growth performance and the blood biochemical values in broilers. *Asian-Aust J Anim Sci* 18:66–74.